STUDIES ON ARTHROPOD CUTICLE. II. ELECTRON MICROSCOPE STUDIES OF EXTRACTED CUTICLE [1, 2, 3]

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In previous work with the electron microscope it was found that sections of cockroach cuticle showed alternating denser and less dense laminae (Richards and Anderson, 1942a). Following treatment with hot alkali solutions this density differentiation was lost. Several possible explanations were suggested in the above paper, the two chief ones being that either a localized heavy component (e.g., protein) in the dense laminae was removed, or that the alkali treatment not only removes certain components but also causes or is followed by a redistribution of the remaining components (at least principally chitin). The x-ray diffraction studies of Fraenkel and Rudall (1940, 1947) suggest that the latter explanation is more probably correct. The present studies were undertaken to clarify this point further and to evaluate the use of isolated cuticles in permeability studies.

In general, we have found that any treatment leading towards chitin purification alters the membrane structure extensively. It follows that the extraction method of analysis has little value for a study on localization of components in the cuticle, that the cuticle cannot be viewed as a fixed framework of chitin micelles in which the other components are embedded, and that purified chitinous membranes, such as used by Yonge (1936) and Alexandrov (1935), are without biological significance for studies on the permeability of arthropod cuticle although normal isolated cuticles may give valid data. Unexpected variations between purified chitin membranes from different sources were found; the significance of these variations is discussed.

Materials and Methods

In our electron microscope studies on arthropod cuticle we have found it most satisfactory to perform the bulk of the experimental and observational work on readily prepared thin membranes. Subsequently results are checked by examination of other cuticular membranes and, when necessary, with sectioning and other ancillary methods. Accordingly the bulk of the work recorded in the present paper was done with large tracheae from a cockroach (*Periplaneta americana*). These tracheae contain chitin as well as protein, polyphenol and lipid components. Data obtained with cockroach tracheal membranes were then checked with crop linings,

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[3] Acknowledgment is due to the Electron Microscope Committee of the University of Minnesota for making the instrument available to us, and to Miss Virginia Kletzin for technical assistance.

rectal linings, soft general body cuticle, wing membranes, various types of scales and setae, and other tracheal membranes including ones consistently negative to chitin tests. The work recorded in the present paper was restricted to membranes sufficiently thin for direct electron micrography.

Normal structure was determined from membranes dissected out in saline solution, rinsed briefly in distilled water, and then placed on electron microscope screens and air-dried (Richards and Anderson, 1942 a, b, c; Richards and Korda, 1947, 1948). Tests on series of specimens treated with distilled water for periods ranging from several minutes to several hours at room temperatures showed no significant differences as far as can be detected with electron micrographs; if the brief rinse in distilled water necessary to clean the surface of the tracheal membranes has any effect on the membrane itself we were not able to detect it (see also under water extraction experiments in main section of this paper). The larval cuticle of mosquitoes and membranes from the gut were wiped gently with a soft brush to facilitate cleaning. The mosquito wing membranes were mounted directly without any treatment except the indicated experimental treatments. The membranes used in the present studies are reasonably large; accordingly they were placed directly onto the screens used in electron micrography (i.e., no supporting membrane of collodion, formvar, etc.) and the membrane under consideration is the only membrane in the field.

Experimentally treated membranes were handled in the same manner except for receiving the indicated treatments. In all experiments, a piece of membrane was removed from the animal, divided into two or more pieces, one of which was always mounted as control for the other piece or pieces which were given treatments. All tests recorded were run in duplicate, and many of them were repeated.

In electron microscope work on arthropod cuticle it is always necessary to consider the possibility of instrumental errors, especially the destructive effects of electron bombardment and of heat produced thereby (Richards and Anderson, 1942a; von Borries and Glaser, 1944). The more gross effects recorded in the present paper can be readily seen by dark-field microscopy or phase-contrast microscopy in membranes still in the solutions used. They are therefore not produced by either the drying or the electron bombardment—although it is quite possible that some of them may be augmented by these factors. The similar appearing but more minute effects cannot be observed directly with a light microscope but it seems reasonable to presume that those shown in the present paper are, like the gross effects, produced by the chemical treatment because birefringence values do not change significantly on drying.

As is generally known, one may remove from insect cuticle (a) the water, (b) the lipids, (c) the water-soluble protein, or (d) presumably all the components other than chitin.[4] No method is known for the removal of chitin without the simultaneous complete dissolution of the membrane. Since electron micrography must be performed in a vacuum, it has not been possible to study hydrated membranes. Treatments used were selected from the literature, and were chosen to cover the various methods that have been used for the removal of certain components, especially those used for the purification of chitin.

[4] Calcium salts which make up so large a part of many crustacean cuticles are absent from almost all insect cuticles (absent from all of those studied by us).

Table of Treatments Employed

Agent	Strength	Temp. (°C.)	Time	Type of cuticle
KOH	5%	20	6 + 10 days	C.T.
	5%	100*	1 day	C.T., M.W.
	20%	25	2 days	M.L.C.
	20%	65	2 days	M.L.C.
	20%	85	4 hours	C.T.
	20%	100*	1 day	M.W.
	40%	100*	10 minutes	L.P.M.
	conc. (= ca. 60%)	160*	15 min. to 2 hrs.	C.T., C.H.G., C.C., Ce.S., L.C., M.W., M.L.C., (H.B.A.S.)†
NaOH	5%	20	2–12 days	C.T., M.L.C., H.C., M.L.T., Ce.S.
	5%	65	6 + 10 days	C.T.
	5%	100*	5 + 9 days	C.T.
	10%	100*	1 day	(H.B.A.S.)
	20%	85	4 hours	C.T.
HCl	5%	100*	1 day	C.T.
Pepsin (+ HCl)	10%	36	1 day	C.T., L.C., L.P.M., H.B.A.S.
Diaphanol‡	full	25	1, 5, 10 + 41 weeks	C.T., C.C., (H.B.A.S.)
$KMnO_4$	0.1%	20	2 days	C.T.
H_2O_2	30%	20	2 days	C.T.
$CHCl_3$ (through acetone)	C.P.	reflux	5 min. to 2 hrs.	C.T., H.B.A.S.
$CHCl_3$ then pepsin	as above	as above	$CHCl_3$ for 2 hrs. pepsin for 1 day	C.T., H.B.A.S.
Aerosol OT§	0.02%	25	1 day	H.B.A.S.
Isotonic salt sol.	—	25	2–7 days	C.T., H.B.A.S.
Distilled water	—	25	few min. to 2 weeks	C.T., H.B.A.S., M.L.C.
	—	30	1 + 2 days	H.B.A.S., M.W.
	—	60	1, 7 + 14 days	C.T., M.W.
	—	65	1, 2 + 7 days	H.B.A.S., M.L.C.
	—	100*	1, 7, 14 + 28 days	C.T., H.B.A.S., M.W., M.L.C.
	—	reflux	5 days	C.T.

* In sealed glass ampoules.

† Parentheses indicate that the membrane was completely destroyed by the treatment.

‡ Saturated solution of chlorine dioxide in 50 per cent acetic acid.

§ Di octyl sodium sulfo succinate.

C.C. = Cockroach (*Periplaneta americana*), membrane lining crop.

C.H.G. = Cockroach, membrane lining hind gut.

C.T. = Cockroach, trachea (large trachea extending anterior from prothoracic spiracle to head).

Ce.S. = Centipede, sensillae (Richards and Korda, 1947).

H.B.A.S. = Honey bee, abdominal air sac (parentheses indicate destroyed).

H.C. = Housefly, membrane lining crop.

L.V. = Blowfly (*Lucilia illustris*), membrane lining crop.

L.P.M. = *Lucilia*, peritrophic membrane.

M.L.C. = Mosquito (*Aedes aegypti*), larval cuticle of abdomen.

M.L.T. = Mosquito, larval tracheae.

M.W. = Mosquito, wing membrane.

The preparations given lengthy treatments and those treated at higher temperatures were placed in sealed glass ampoules. Most of the hydroxide treatments were given in pyrex glass but a few were run in tubes of fused silica. The preparations from membranes treated with alkali must usually contain a trace of alkali because this is not completely washed out of cuticle by rinsing in water (or even prolonged soaking). Probably more alkali remains in those preparations washed in alcohol. Full removal of the alkali by soaking in one per cent HCl followed by washing in distilled water does not alter the electron microscope picture; accordingly we have usually ignored this trace of alkali that may remain in the preparation (Figs. 5–6 and 10–12 show preparations treated with HCl to remove all the alkali, while Figs. 7–9 were not washed in acid solution). The effects of washing in various ways is discussed in the next section. Diaphanol is an oxidizing agent prepared by saturating a 50 per cent solution of acetic acid with sulfur dioxide; it can be washed from cuticle with water but we used the supposedly gentler treatment (recommended by Koch, 1932) of transferring through 50 per cent, 30 per cent and 10 per cent acetic acid to water, then washing in one per cent $NaHSO_3$ at room temperature or 2 per cent $Na_2S_2O_3$ at 97° C., and finally washing again in water. Preparations treated with potassium permanganate were washed first in water, then in 0.1 per cent $NaHSO_3$, and then again in water. Specimens refluxed with chloroform were transferred from water to chloroform through acetone, and back to water through acetone; they were dried from water. For extraction with aqueous media, double distilled water was used, the second distillation being made in a pyrex glass still.

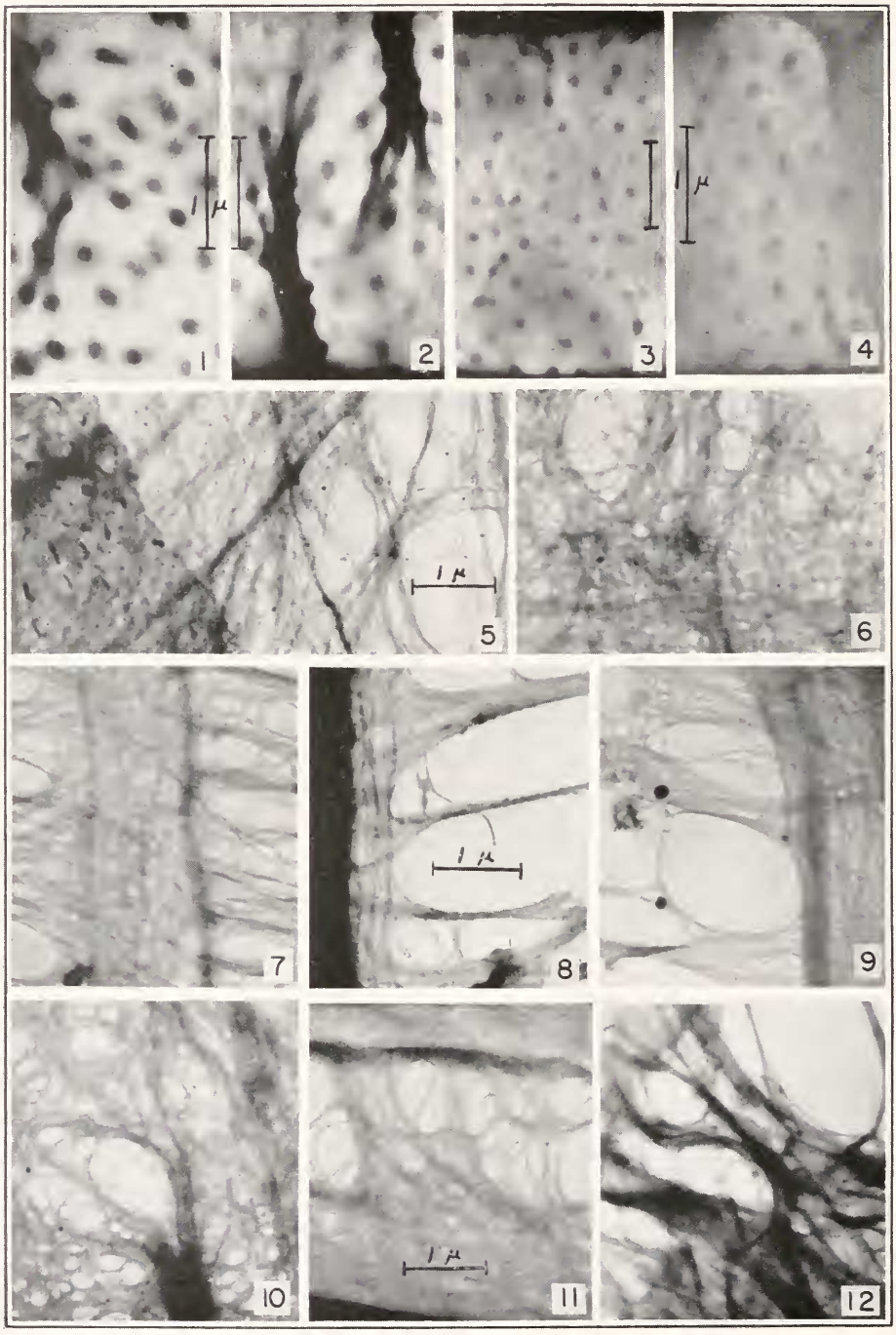
The electron microscope used was an RCA model EMU which uses an accelerating potential of 50 kilovolts. Most of the present work was done with the standard instrument as received from the factory. More recent pictures (those bearing numbers above 300) were taken after modifying the instrument by installing a saturation emission type electron gun and compensating the objective lens and after introducing the recently developed operational refinements to increase resolution (Hillier and Ramberg, 1947). Electron micrographs were made at an initial magnification of 6,000–10,000 and photographically enlarged to the size used for reproduction. Resolution in most of the figures of thin membranes presented is better than 0.01 μ; in some of the best it is of the magnitude of 0.005 μ; resolution in thicker membranes (wings, mosquito cuticle) is less good (probably 0.02–0.05 μ).

The Normal Structure of Arthropod Cuticle

Only a cursory summary to provide background for the following sections will be given. In current terminology the cuticle is divided into two major subdivisions distinguished by the presence and absence of the polysaccharide chitin. The outer layers of the cuticle which contain no chitin are termed "epicuticle," and the inner layers composed of a laminated chitin and protein matrix are termed "endocuticle." The epicuticle is usually distinctly double, consisting of an outer "lipid epicuticle" and an inner "protein epicuticle" (Richards and Anderson, 1942a; Dennell, 1946; Wigglesworth, 1947). Numerous further subdivisions are commonly recognizable but the above three will suffice for the purposes of the present paper.

The above three subdivisions are not necessarily all present. The cockroach has all three subdivisions in the thick cuticle covering the outside of its body and

PLATE I



also in the very thin cuticle covering its crop and its tracheae (despite the fact that these membranes are only in the range of 0.01 to 0.1 μ thick when dry). The same is true for wing membranes. Using the above terminology, the air sac and tracheae of honey bees consist only of epicuticle but have both lipid and protein subdivisions of this. The abdominal cuticle of mosquito larvae lacks a demonstrable lipid layer; it may be said to consist of a protein epicuticle plus an endocuticle. The peritrophic membrane (in the midgut) is a special case due to its peculiar origin; it contains both protein and chitin (Wigglesworth, 1939). For further analysis see papers cited in the bibliography.

Trachea are tubular invaginations of the body wall. In keeping with their origin they possess a secreted cuticle which lines the inner surface of the tubes. This cuticle is continuous with the cuticle of the external surface of the body, and the two are generally considered to be of homologous composition. These thin cuticular tubes are made more rigid by the development of supporting helical thickenings in the endocuticle. The helical thickenings are called taenidia. In large tracheae the taenidia are rather thick—too thick for adequate penetration by a 50 kV electron beam—but the membrane between the taenidia is usually quite thin (after drying ranges between 0.01 and 0.05 μ thick in species treated herein) and can be readily examined with an electron microscope (Richards and Anderson, 1942 b, c). Commonly, after removal of part of the material the taenidia decrease in density sufficiently for adequate electron penetration (Figs. 7, 17, etc.).

The intertaenidial membrane is seldom of uniform composition (Richards and

PLATE I

Cockroach (*Periplaneta americana*), large tracheae from prothorax

FIGURE 1. Normal; intertaenidial membrane. The spots are thickenings in the endocuticle. (No. 126e) 16,000 ×.

FIGURE 2. Normal; intertaenidial membrane with edge of taenidium at lower margin; bars are braces extending from taenidium onto membrane. (No. 121d) 16,000 ×.

FIGURE 3. Normal; intertaenidial membrane with edges of taenidia at upper and lower margins. (No. 66c) 12,000 ×.

FIGURE 4. Washed with acetone, then refluxed with boiling chloroform for 5 minutes. (No. 178d) 16,000 ×.

FIGURE 5. Treated with 5 per cent KOH at 20° C. for 6 days, washed in 1 per cent HCl, then water. At left is broken, partially disintegrated epicuticle, at right only endocuticle. (No. 48c) 12,000 ×.

FIGURE 6. Treated with 20 per cent KOH at 85° C. for 4 hours, washed in 1 per cent HCl, then water. Only endocuticle of membrane. (No. 66d) 12,000 ×.

FIGURE 7. Treated with conc. KOH at 160° C. for several hours, washed in water. Taenidium vertically through center of picture, intertaenidial membrane on both sides. Resolution relatively poor. (No. 14c) 12,000 ×.

FIGURE 8. Treated with conc. KOH at 160° C. for 20 minutes, washed in water. Example with larger holes. (No. 19a) 12,000 ×.

FIGURE 9. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in alcohol. Taenidium placed vertically on right, membrane on left. (No. 198c) 12,000 ×.

FIGURE 10. Treated with 5 per cent NaOH at 20° C. for 10 days, washed in 1 per cent HCl, then water. Intertaenidial membrane only, with partially disintegrated brace extending from lower center upwards. (No. 46b) 12,000 ×.

FIGURE 11. Treated with 5 per cent NaOH at 65° C. for 6 days, washed in 1 per cent HCl, then water. Intertaenidial membrane only. (No. 32c) 12,000 ×.

FIGURE 12. Treated with 20 per cent NaOH at 85° C. for 4 hours, washed in 1 per cent HCl, then water. Intertaenidial membrane only. (No. 59e) 12,000 ×.

Korda, 1948). It usually either is studded with thickenings in the endocuticle (Figs. 1–4) or contains a reticulate meshwork of thickenings (Figs. 45–56). The structural details revealed in electron micrographs of tracheal membranes show more variability than one would like for precise quantitative work, but the changes discussed are so great in comparison to this variability that we may, as far as the present paper is concerned, treat the normal membrane structure as though constant.

Effects of Lipid Extraction

The lipid epicuticle is not ordinarily detectable in mounts of entire membranes because it is so delicate (and seemingly so uniform) that it is lost against the stronger background of protein and chitin-protein layers. To demonstrate it convincingly with an electron microscope one has to isolate the layer (Richards and Anderson, 1942a). The figures given in the present paper show only the protein epicuticle and the endocuticle even when the lipid epicuticle is still present.

As can be seen from Figures 4 and 48, treatment with acetone and boiling chloroform produces no distinct changes in either the protein epicuticle or the endocuticle. Seemingly the effect of lipid solvents is indeed limited to removal of the superficial lipid layer, as Wigglesworth (1945) and Beament (1945) assumed.

Effects of Chitin Purification

Extensive studies were made on the effects of chitin purification by various methods with the large tracheae from the prothorax of the cockroach as standard test material (see table). The chitin pictures obtained with this particular material were strikingly similar irrespective of the chemical method used for purification, irrespective of whether the purification was partial or what is referred to in the literature as complete, and irrespective of whether the chitin molecules were left unchanged or converted into chitosan (Figs. 5–23).

Figure 5 represents an incomplete purification with alkali. At least it is incomplete in the sense that the protein epicuticle is not completely destroyed; the epicuticle is clearly discernible in somewhat altered but recognizable condition over the left-hand part of the figure. Since it is not feasible to strip all the epicuticle off manually and test the endocuticle for proteins it is not possible to say whether the fibrous meshwork is pure chitin or still contains some protein. Ninhydrin and xanthoproteic tests on such membranes are positive but this might be due solely to the only partly disintegrated protein epicuticle. Figures 9 and 10 are other presumably incomplete chitin purifications with alkali, while Figures 6, 11 and 12 are supposedly complete chitin purifications. Figures 7 and 8 represent membranes in which the chitin has been changed into the de-acetylated form, chitosan. This picture is not significantly changed by staining with I + KI with or without a subsequent rinsing in one per cent H_2SO_4 (chitosan color test) or by prolonged soaking in a solution of potassium iodide. Figures 13–18 and 20 represent unknown degrees of purification with diaphanol, hydrogen peroxide, potassium permanganate, hydrochloric acid and pepsin. Figures 14 and 15 are probably pure or nearly pure chitin, the other probably only partially purified.[5] A

[5] We have experienced difficulty in obtaining negative ninhydrin tests on these membranes, even ones which considering the treatment should be highly purified chitin. Commonly we obtained negative xanthoproteic and Millon tests and yet a moderate positive ninhydrin test.

more obscure fibrous pattern is shown by the endocuticle in Figure 23 where the treatment with hot water can scarcely have given more than a partial purification to judge from comparison with the data of Fraenkel and Rudall (1940).

In all of these cases, whatever the degree of purification, the endocuticle of cockroach tracheae became primarily a fibrous meshwork (except for a delicate basal sheet to be discussed later). This is clearly not its normal state. Pictures of tracheae which have been deliberately abraded show areas where the epicuticle has been manually torn from the underlying endocuticle; in these cases the endocuticle appears as a true sheet without any discernible fibrous structure (Fig. 21). Also, so gross a fibrous structure would be discernible through the epicuticle in the control preparations (as it is in Fig. 23). Accordingly, we must conclude that the fibrous mesh is produced by the treatment.

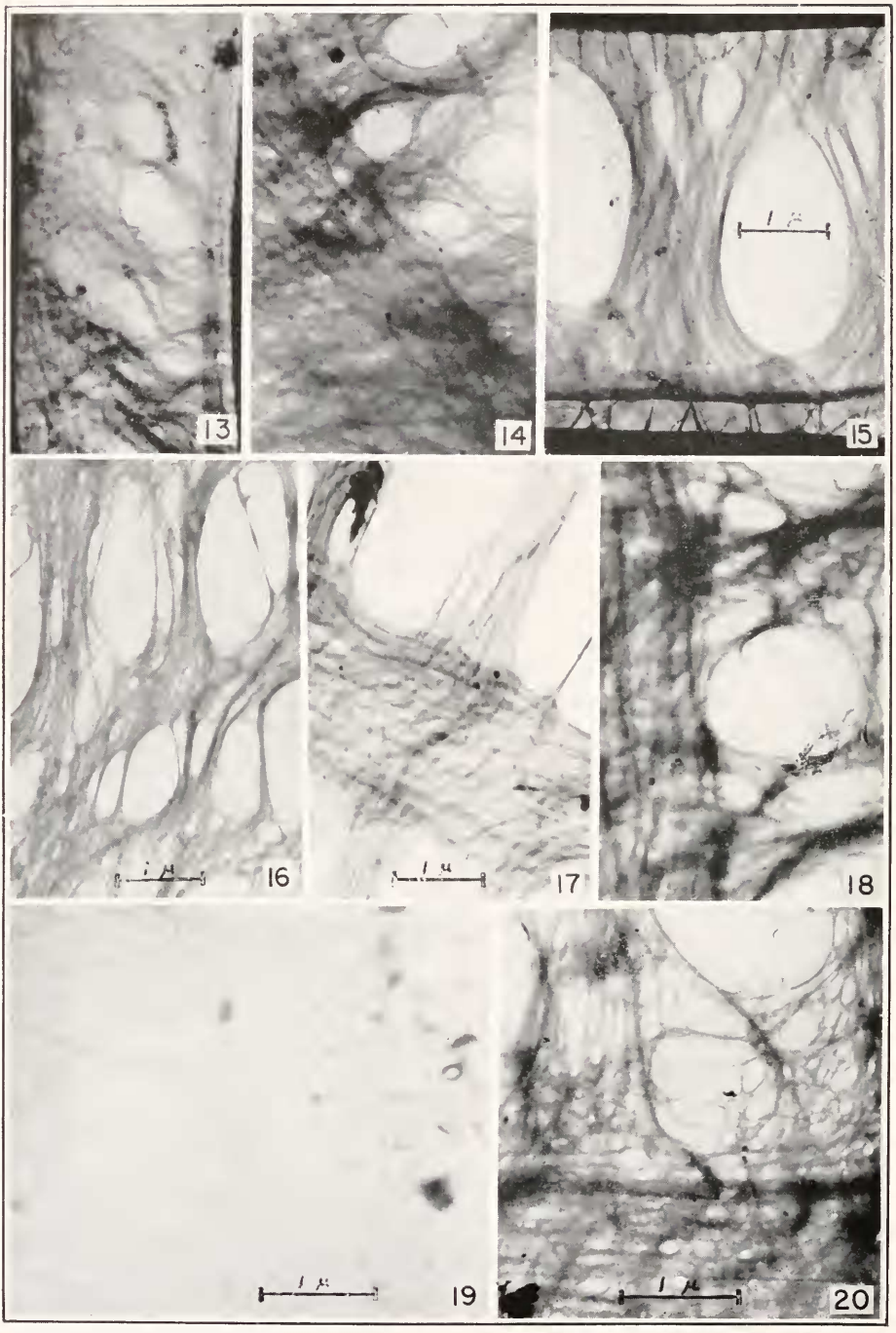
As can be seen from the pictures given, larger fibers can usually be resolved as multiples of microfibers. The microfibers of chitin are not of constant size, but variation among them does fall within a fairly narrow range in both tracheae and other membranes. Most of the microfibers have diameters in the range of 0.01 to 0.03 μ (100–300 Å) when dry. A few less than 100 Å in diameter were estimated as approximately 75 Å. The fact that in other membranes (see below) microfibers of similar diameters may be oriented randomly suggests that there may be some real significance to these dimensions. Perhaps the microfiber diameters ($< 100 - 300$ Å) represent the range of micelle or crystallite dimensions (the a and c axes thereof) but we have no proof of this. The report by Clark and Smith (1936) of the preparation of a "micellar solution" of chitin makes more plausible the suggestion that micelles might orient into microfibers of micellar diameters on removal of other membrane components. The fact that the diameters (1 μ) of the natural fibrils teased from lobster tendons by Clark and Smith are many times larger than our chemically prepared microfibers does not invalidate the above suggestion.

The chitin microfibers do not make a constant pattern except that in general they run longitudinally in the taenidia and at a right angle to this in the membrane between taenidia (Figs. 7–9, 17, 18, 20). Usually a finer reticulation is produced from gentler alkali treatment (lower concentrations and lower temperatures).[6] What seems more significant in this respect is that the pattern developed depends to some extent on the method used to remove the alkali. This effect of washing was studied in greater detail with other membranes (see below) but the generality that more destruction and coarser structure results from washing in alcohol can be seen from comparison of Figures 9 and 10. It does not seem likely, therefore, that the chitin molecules are arranged as shown in Figures 5–20 in the normal tracheal endocuticle. It seems more reasonable to assume that the chitin molecules are

It is well known that ammonia and certain amines interfere with the ninhydrin test. Dr. W. M. Sandstrom of the Chemistry Department tells us that it would not be surprising if chitin gave a positive ninhydrin test, especially if de-acetylated. We have had to conclude that the ninhydrin test is not reliable for determining the removal of protein from chitin. Unfortunately most of our preparations were made and tested before we were aware of this, and accordingly we do not have as accurate information as we would like on the membranes used for electron microscope examination. However, subsequent tests using the same procedures indicated that the remarks in the text about purification are reasonably accurate.

[6] This is not apparent from the figures because mostly micrographs showing finer fibers and finer holes were chosen as illustrations.

PLATE II



arranged otherwise in the intact endocuticle, that they are held in place by the protein molecules, and that on removal of the protein they reorient themselves into these micro fibers which run parallel to the surface of the membrane (both before and after drying). This latter interpretation is in fair agreement with the x-ray diffraction data of Clark and Smith (1936) and Fraenkel and Rudall (1940).

Some explanation is required, however, for the consistent tendency mentioned above for the microfibers to run longitudinally in the taenidia and at a right angle to this between taenidia. The fibers in the intertaenidial membrane appear to cross the taenidia (Fig. 17), and actually a continuous fibrous membrane can be separated from the taenidia after drastic alkali treatment. It seems therefore that there is a continuous endocuticle with fibers of one orientation and attached to this the taenidia with fibers set at a right angle to those in the membrane. Both sets are parallel to the surface of the membrane, and both are distinct from the more delicate homogeneous sheet to be discussed in the next paragraph. Since chitin is an anisotropic substance it is possible to determine that there is a corresponding difference in the orientation of the chitin micelles. Using a polarized-light microscope and an appropriate compensator we have shown that when a trachea is oriented at 45° to the crossed Nicols, the taenidia are in the retarding orientation when the intertaenidial membranes are in the accelerating orientation, and vice versa. This is true for normal tracheae as well as for ones treated with alkali to purify the chitin or convert it to chitosan (amplitude of birefringence much greater after alkali treatment). It follows from this that the chitin micelles in the intertaenidial membranes are indeed at right angles to those in the taenidia.[7] This point, which is of little importance to the present discussion, will be treated further in a subsequent paper devoted to the structure of tracheal cuticles (Richards and Korda, 1948).

[7] Picken, Pryor and Swann (1947) record a mosaic of fibers for the chitin fraction of *Donacia* cocoons. While the details remain to be proven it is simple enough to rationalize such differences between tracheal and cocoon membranes on the basis of origin.

PLATE II

Cockroach (*Periplaneta americana*), large tracheae from prothorax

FIGURE 13. Treated with diaphanol at 25° C. for 1 week. Intertaenidial membrane; edges of taenidia at sides. (No. 50c) 12,000 ×.

FIGURE 14. Treated with diaphanol at 25° C. for 5 weeks. Taenidium and intertaenidial membrane. (No. 61a) 12,000 ×.

FIGURE 15. Treated with diaphanol at 25° C. for 5 weeks. Intertaenidial membrane with edges of taenidia at top and bottom. (No. 60a) 12,000 ×.

FIGURE 16. Treated with 30 per cent hydrogen peroxide at 20° C. for 2 days, washed in water. Intertaenidial membrane only. (No. 43b) 12,000 ×.

FIGURE 17. Treated with 0.1 per cent potassium permanganate at 20° C. for 2 days. Taenidium and intertaenidial membrane. (No. 41e) 12,000 ×.

FIGURE 18. Treated with 5 per cent HCl at 100° C. for 1 day (in sealed glass ampoule), washed in water. Taenidium on left, membrane on right. (No. 63c) 12,000 ×.

FIGURE 19. Epicuticle manually separated after treatment of trachea with 10 per cent pepsin in acid solution at 36° C. for 1 day, washed in water. Note differences in structure corresponding to taenidial and intertaenidial regions (see Fig. 20). (No. 58c) 16,000 ×.

FIGURE 20. Endocuticle manually separated after treatment of trachea with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. Taenidium at bottom, intertaenidial membrane above. (No. 58e) 16,000 ×.

In addition to the two sets of fibrous sheets treated in the preceding paragraph there is a continuous sheet in these purified tracheae. This continuous sheet is so thin and, more to the point, so homogeneous that it was overlooked by us for a long time. It is not discernable in any of the pictures reproduced here although it is present in Figures 8, 9 and some others. It is discernable in the occasional cases when it breaks after long bombardment. Once its presence was suspected it was readily demonstrated by placing small particles on it (e.g., a deposit of magnesium oxide smoke). This homogeneous sheet does not seem to be any part of the epicuticle because after separation of epicuticle from endocuticle by pepsin digestion it is found intimately associated with the chitin microfibers in the endocuticle. Likewise it does not seem possible for it to be the basement membrane of the tracheal epithelium because of alkali resistance, different appearance from known preparations of the basement membrane and intimate association with the fibrous endocuticle. It is not present in all preparations but we interpret this as probably meaning that it is commonly lost in the course of preparation. We know only three things about this membrane: its resistance to pepsin and hot alkali, its homogeneity (at least when dry) even in pictures where the resolution is to better than 50 Å (0.005 μ), and its intimate association with microfibers of the intertaenidial endocuticle. It is certainly a very thin membrane (probably less than 100 Å thick when dry) but we do not have pictures which permit an estimation of its thickness. Perhaps it is a sheet of chitin but one would have to obtain this sheet free from the known chitinous fibers before reliance could be placed on the chitin color tests. Further work is needed on the nature of this sheet.

The protein epicuticle is destroyed by the various treatments used. However, if the treatment is slight enough to give only partial purification, a reasonably normal protein epicuticle may be peeled off the modified endocuticle. This is shown particularly well by Figure 19 (which was stripped from the preparation that gave Figure 20). This epicuticle (and also the bee air sac) never shows a fibrous structure such as the endocuticle of cockroach tracheae does. In the early stages of purification the endocuticle is more readily altered than is the protein epicuticle. This suggests that the protein is more easily removed from the chitin-protein binding than from membranes where protein chains are linked directly to one another. Otherwise stated, it would seem that, if we assume only one protein species is involved, the cuticular proteins of cockroach tracheae are less strongly bound when linked to chitin than when linked to other protein molecules.

Similar but less extensive studies were made with a number of other types of cuticle. In single tests similar results were obtained with tracheae of *Blatta orientalis* and *Galleria mellonella*, but tracheae of *Xcodiprion lecontei* became only vaguely fibrous, and those of *Calandra oryzae* retained their normal appearance. The cuticle lining the crop (part of fore gut) of adult flies gave results essentially similar to those from roach tracheae.[8] On treatment with pepsin the crop membrane of a blowfly developed numerous small holes and a vague fibrous structure (Fig. 28). On treatment with hot concentrated alkali it developed a heavy fibrous mat in which the relative vagueness of the individual fibers is at least partly due

[8] Considerable difficulty is encountered in getting gut membranes sufficiently clean for electron microscopy. Controls are only seldom clean enough to permit comparison with treated preparations, and then usually only clean in spots. Blurred areas on Figures 27 and 30 are interpreted as adhering debris.

to the decreased resolution occasioned by thickness of the preparation (Fig. 29). It seems that the crop membrane differs from the cockroach tracheae principally in having the chitin fibers oriented randomly and in requiring somewhat more drastic chemical treatment for purification. The crop lining of the closely related housefly gave similar results: mild alkali treatment gave relatively little change and only a very vague fibrous appearance when the alkali was removed with water (Fig. 31); removing the alkali with alcohol did not increase the fibrous appearance but, as usual (see below), resulted in a less normal appearing membrane with numerous minute holes (Fig. 32).

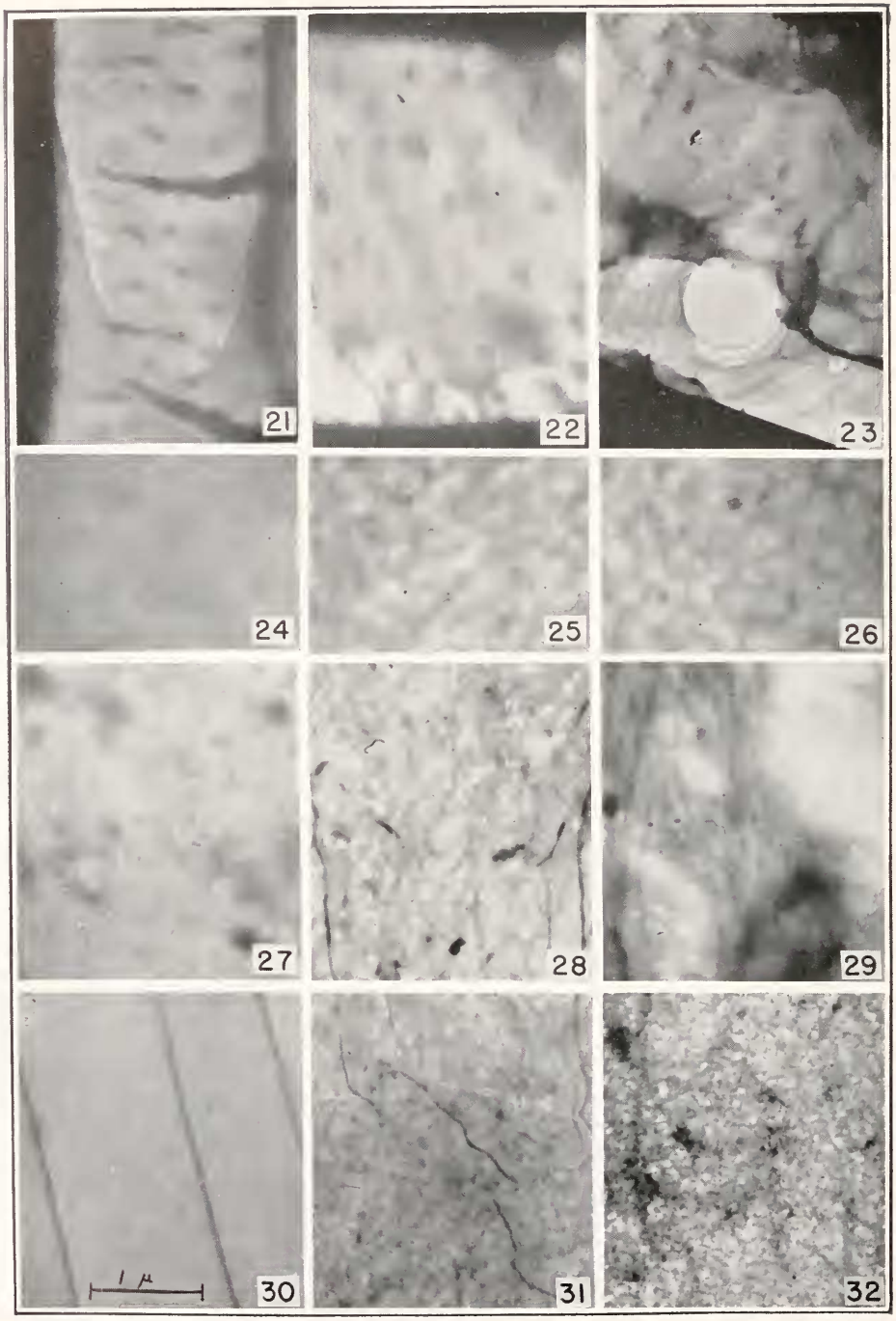
The heavier membrane lining the crop of the cockroach is more resistant. Ignoring the heavy, spine-like projections and considering only the homogeneous membrane of the controls (Fig. 33), we find an irregular removal of material by hot concentrated alkali (Fig. 34). This alkali treatment was quite drastic and is commonly thought to remove all components other than chitin and also to convert all the chitin to chitosan. Yet the membrane is still intact and shows islands of greater density. Prolonged treatment with diaphonal (41 weeks) gave a different picture in which a vague, unoriented fibrous structure is apparent, with minute holes visible in the thinner parts (Fig. 35).

The peritrophic membrane of the midgut of adult flies gave pictures not encountered with any other membrane examined. This membrane has a different origin from ordinary cuticle but agrees with endocuticle in being composed of chitin and protein (Wigglesworth, 1939). Sufficiently clean controls show a seemingly homogeneous membrane (Fig. 24). Treatment with pepsin and alkali gives the same altered picture (Figs. 25–26); an intact membrane with relatively gross, denser reticulations which presumably represent reticulate thickenings (reminiscent of the reticulations seen in the normal intertaenidial membranes of many tracheae, compare Figure 55).

Wing membranes of adult mosquitoes showed the least alteration of any illustrated in this paper. Mild alkali treatment gave no detectable change (Fig. 36). Drastic alkali treatment resulted in the production of some vesicles (Fig. 37) or actual holes (Fig. 38). None of the treatments revealed any fibrous meshwork. Equally great resistance to alkali treatment was shown by centipede sensillae (Richards and Korda, 1947) and by strongly "chitinized" butterfly scales (unpublished), and by the large tracheae of the beetle, *Calandra oryzae*. These showed only a considerable decrease in density.

The cuticle of mosquito larvae has already been fractionated and studied (Richards and Anderson, 1942a). Some of these experiments were repeated especially to study the effects of various types of washing following alkali treatment. In preparing alkali-treated membranes for permeability studies Yonge (1936) washed in 95 per cent ethyl alcohol and then transferred to water for permeability tests. He assumed (erroneously) that the alkali treatment removed only the epicuticle but did not affect the endocuticle. Normal structure of mosquito larval cuticle is shown in Figure 39; it shows a fairly uniform membrane crossed by smooth parallel ridges approximately one micron apart. After an alkali treatment that should leave pure or nearly pure chitin, diverse pictures are obtained depending on the washing procedure. Washing in water results in a membrane with numerous, rather crowded, lighter spots that seem to represent vesicles in the membrane (Fig. 42); one could interpret this as representing randomly oriented vague

PLATE III



chitin microfibers. If the treated membrane is washed in alcohol and then in water, a vague but distinct fibrous appearance is obtained (Fig. 40). If the treated membrane is washed only in alcohol many holes but no distinct fibers result (Fig. 41). If the treated membrane is washed in water first and then in alcohol a more nearly uniform membrane results with only a hazy suggestion of fibrous structure (Figs. 43–44). From this series of experiments, as well as from less extensive experiments with different washing procedures on other membranes (as noted in preceding paragraphs), it is obvious that the structure of the treated membranes is affected not only by the alkali but also by the subsequent treatment. The chitin molecules must be labile indeed for a water treatment to return a membrane such as that shown in Figure 41 to the condition of that shown in Figure 40. It does not seem desirable to attempt interpreting the diverse set of pictures shown in Figures 40–44 other than to say that clearly no one of them is "normal," that no one would have any biological significance if used in a permeability experiment and that washing alkali out with alcohol seems to cause more deviation from normal membrane structure than washing with water does.

Effects of Extraction with Water and Aqueous Solvents

Fraenkel and Rudall (1940, 1947) and others have shown that some of the protein of insect cuticles is extractable with hot water. There is an inverse relationship to sclerotization, i.e., soft, non-sclerotized cuticles lose much of their protein on extraction with hot water, while hard, sclerotized cuticles lose relatively little.

Plate III

All figures reproduced at 16,000 ×

Figure 21. Cockroach trachea. A normal trachea manually abraded. The epicuticle has been torn and peeled away from a small area leaving the intact endocuticle exposed. (No. 384c)

Figure 22. Cockroach trachea. Treated with distilled water at 100° C. for one week. (No. 336e)

Figure 23. Cockroach trachea. Treated with distilled water at 100° C. for 2 weeks. Area of approximately maximum effect. (No. 340d)

Figure 24. Blowfly (*Lucilia illustris*), peritrophic membrane. Normal, washed in water but probably not entirely clean. (No. 89e)

Figure 25. Blowfly, peritrophic membrane. Treated with 40 per cent KOH at 100° C. for 10 minutes, washed in water. (No. 87b)

Figure 26. Blowfly, peritrophic membrane. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 78b)

Figure 27. Blowfly, crop lining. Normal, washed in water but not entirely clean. (No. 79b)

Figure 28. Blowfly, crop lining. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 80c)

Figure 29. Blowfly, crop lining. Treated with conc. KOH at 160° C. for 15 minutes, washed in water. (No. 79d)

Figure 30. Housefly (*Musca domestica*), crop lining. Normal, washed in water but speckles may be debris. (No. 193c)

Figure 31. Housefly, crop lining. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in water only. (No. 194a)

Figure 32. Housefly, crop lining. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in 95 per cent ethyl alcohol. (No. 195a)

Since isolated cuticles are sometimes used in permeability studies, in our laboratory and elsewhere, it seemed desirable to see what we could learn from electron micrographs of membranes extracted with water and salt solutions at various temperatures.

Cockroach trachea soaked in distilled water at 25° C. for periods up to two weeks appeared normal. Ones soaked at 60° C. for one and two week periods seemed to be somewhat affected but the differences from control preparations were not well marked. Ones soaked at 100° C. showed a clear loss of substance, development of a network type endocuticle with some fibrillar structure, and a considerable tendency for the epicuticle to break (Figs. 22–23). Cockroach tracheae exhibit so much variability that they are useful only when the effects are striking (as in chitin purifications) and accordingly it is not safe to draw conclusions from the negative results of extraction with water at low temperatures.

The air-sacs of honey bees gave more clearly defined changes and so were studied rather intensively.[9] As far as can be ascertained from structure visible in the electron micrographs, soaking these membranes in distilled water at 25° and 30° C. for periods up to a week produces no change. At the higher temperatures which Fraenkel & Rudall used for extracting proteins from cuticles, the effects are striking. Air-sacs treated in distilled water at 65° C. for two days show obvious swelling of the ridges accompanied by decrease in sharpness and density (Fig. 49). Electron micrographs of air sacs treated with hot water do not always give this particular type of picture but do always contrast with control preparations. Another type of picture is shown by Figure 50, of a specimen soaked at 65° C. for a week; in this case the removal of material and the change has not been uniform over the preparation—as can be seen by comparison of right and left halves of this picture. Treatment with distilled water at 100° C. gives still greater change. After one day there is a general fuzziness suggesting removal of material from the ridges (Fig. 51). After eight days the membrane is much more delicate, the ridges and reticulations have mostly disappeared and the membrane between ridges is less dense and therefore thinner (Fig. 52). After a month soaking at 100° only a very delicate, but still continuous, membrane remains (Fig. 53).[10] The delicate membrane remaining in Figure 53 does not show properties of the lipid epicuticle, i.e., it does not disintegrate under electron bombardment; accordingly it may be presumed to represent one of the protein layers. These hot water extractions of air sacs show, as one might have predicted from the work of Beament (1946) and Wigglesworth (1947), that the protein is more readily removed from some portions than from others; seemingly protein is removed most readily from the thickenings and less readily from some of the continuous layers than from others. Whether there are as many discrete layers in the bee air sac as in the thicker epicuticles studied by Beament and Wigglesworth is not known but analysis of these extraction pictures suggests that more than one protein layer is present. This is also suggested, but not proven, by the membranes remaining after pepsin digestion (Figs. 54–55).

The resolution shown in electron micrographs of bee air sacs is excellent.

[9] One should remember that the air-sacs of honey bees are consistently negative to chitin tests.

[10] For focusing electron micrographs some contrast is needed. With such homogeneous membranes this is most readily accomplished, as was done here, by finding an area where minute particles of debris are present.

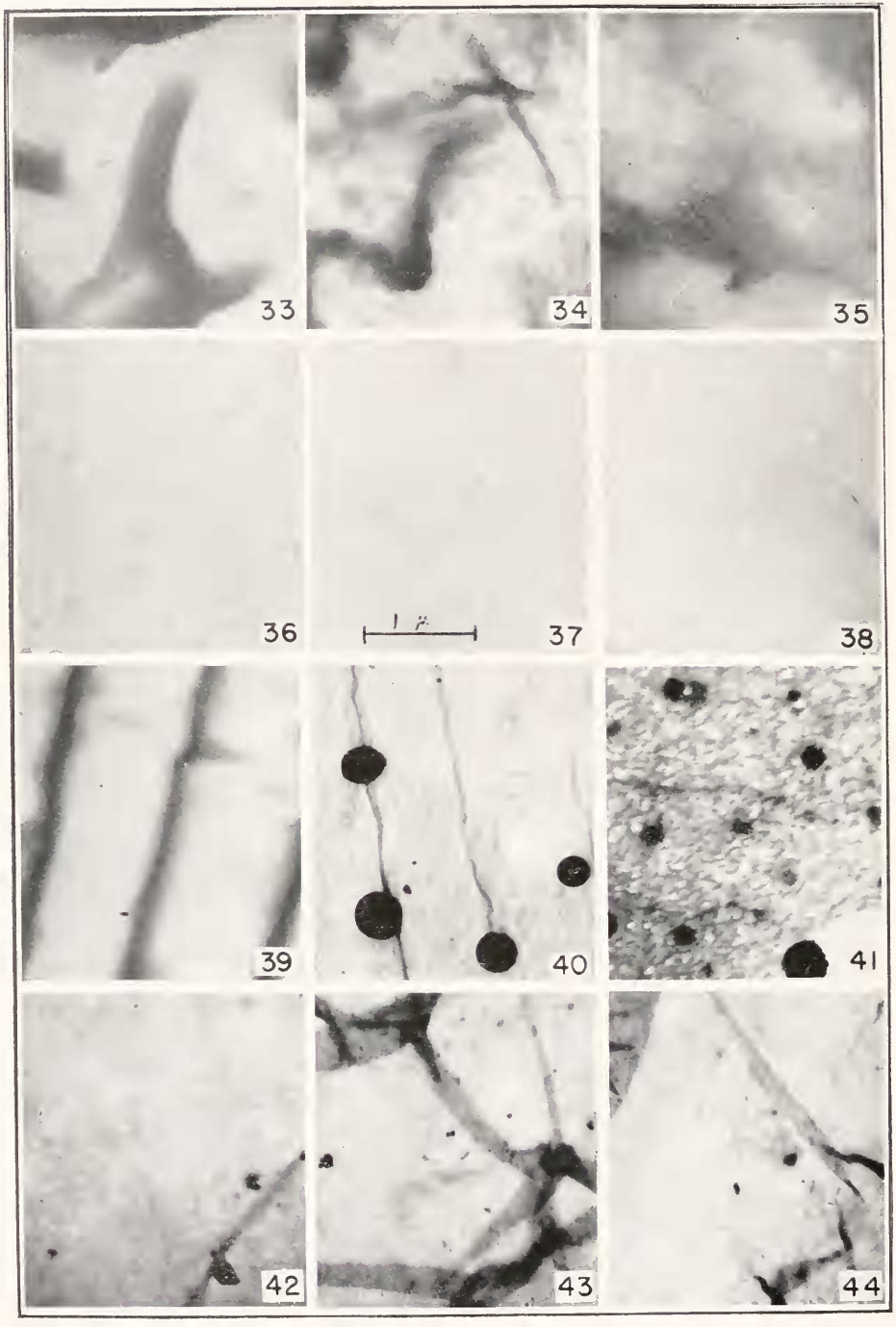
Sharp discontinuities of the diameter of 100 Å should be clearly visible, and in the best pictures sharply contrasting discontinuities (e.g., holes) of half this size should be detectable. However, since we are dealing with a multiple membrane where effects in one layer may be partially hidden by an overlying layer we cannot claim to have proven the absence of effects of this magnitude. To be on the conservative side we will only say that there seem to be no changes down to the range of several hundred Ångstrom units. One could therefore claim that there is good evidence that these air sacs could be used as isolated membranes for permeability studies at room temperatures. At high temperatures they are altered but at room temperatures even rather lengthy treatments give either only exceedingly slight alterations (beyond detection by the present electron micrographs) or else slight effects which are completely masked by drying.

Soaking honey bee air sacs in physiological saline solutions for several days at 25° C. likewise seems to cause no detectable effects (Fig. 48). The same may be said for dilute solutions of some single salts (e.g., KCl). However, it seems likely that strongly hypertonic salt solutions do affect these membranes deleteriously. The effects of hypertonic saline solutions are only slight; an adequately extensive analysis of them has not been completed. We can safely record, however, that strong aqueous solutions of agents commonly used to facilitate protein extraction can affect the structure seen in electron micrographs of bee air sacs (e.g., 6M urea, 5 per cent sodium salicylate, detergents). A number of detergents (anionic, cationic and non-ionic) have been tested in a preliminary manner. Different detergents do not give the same degree of effect but even in dilute solutions they show a considerable disruptive effect (Fig. 56). This fact is of some interest because Wigglesworth (1945), in studying cuticle permeability, compares the effect of removing the lipid epicuticle by the use of lipid solvents with the presumed disruption of the lipid epicuticle by the use of detergents. Clearly this comparison is not a simple one because we can demonstrate no effect for lipid extraction other than the removal of the lipid epicuticle whereas detergents disrupt both the lipid epicuticle and at least some of the protein components of the underlying membranes.

The wings of mosquitoes remain intact after washing in distilled water at various temperatures for various periods. Illustrations are not given. The appearance of normal and control membranes is as shown in Figure 36. After soaking in distilled water, even at room temperatures, the membranes become less dense; this is shown particularly by an increase in contrast between the general membrane and the small darker (denser) areas.[11] Such increased contrast and decreased density is more marked with wing membranes soaked in hot water. It seems that some of the diffuse material is removed by water. Interpretation of the decreased density shown by electron micrographs of wing membranes is difficult. Resolution in these thick, multilayered membranes is relatively poor even in the best pictures, and accordingly any structural alteration would have to be relatively gross to be detected (at least several hundred Ångstrom units). Furthermore, wings consist of two membranes separated by the remnants of dead epithelial cells; it is conceivable that the material removed by water is largely this cellular debris. As far as can be determined from our pictures the wing membrane maintains its integrity. What

[11] The identity of these small dark areas has not been definitely established but it seems likely that they represent thickenings in the endocuticle comparable to those in cockroach tracheae.

PLATE IV



can be stated definitely is that these wing membranes do not become altered in water in a manner comparable to or to the extent that cockroach and bee tracheae do.

The soft, non-sclerotized abdominal cuticle of mosquito larvae was tested as an example representing approximately the other extreme from wing membranes. Normal structure is as shown in Figure 39. Soaking isolated, cleaned cuticles for one day or one week in distilled water at 20° C. produced no positively indentifiable alterations but again it should be stressed that resolution in such thick membranes is not very good (probable order of several hundred Angstrom units). Soaking isolated, cleaned cuticles for one day or one week in distilled water at 65° C. produced marked changes; the results were not entirely constant but were all in the intermediate range between patterns shown by Figures 40 and 42. Treating living larvae with water at 65° C. (which is lethal) for a minute and then removing the cuticle and brushing it clean gave preparations indistinguishable from the controls, but treating such intact larvae with water at 65° C. for an hour produced a fibrous appearance comparable to that obtained by soaking isolated cuticles in water at this temperature. Soaking isolated cuticles for one day or one week in distilled water at 95° C. gave pictures more nearly like Figure 44. No interpretation is made for these results, beyond saying that there are obvious structural changes following treating or soaking at 65° and 95° C. but that there are no clearly demonstrable changes following soaking at 20° C.

The above results may be compared with the data published by Fraenkel and Rudall (1940, 1947). Soaking in water at room temperatures causes very little

PLATE IV

All figures reproduced at 16,000 ×

FIGURE 33. Cockroach, crop lining. Normal, washed in water. Dark structures are heavy projections. (No. 81b)

FIGURE 34. Cockroach, crop lining. Treated with conc. KOH at 160° C. for 15 minutes washed in water. (No. 81c)

FIGURE 35. Cockroach, crop lining. Treated with diaphanol at 25° C. for 41 weeks. (No. 251c)

FIGURE 36. Mosquito (*Aedes aegypti*), wing. Treated with 5 per cent KOH at 100° C. for one day. Not significantly different in appearance from control. The scattered darker areas appear consistently on normal and water-washed mosquito wing membranes but their identity has not yet been established. (No. 90d)

FIGURE 37. Mosquito, wing. Treated with conc. KOH at 160° C. for 15 minutes, washed in water. (No. 91b)

FIGURE 38. Mosquito, wing. Treated with 20 per cent KOH at 100° C. for one day, washed in water. (No. 91a)

FIGURE 39. Mosquito larva (*Aedes aegypti*), abdominal cuticle. Normal, washed in water. (No. 372e)

FIGURE 40. Mosquito larva, abdominal cuticle. Treated with 20 per cent KOH at 65° C. for 48 hours, washed in 95 per cent ethyl alcohol, then in water. (No. 368e)

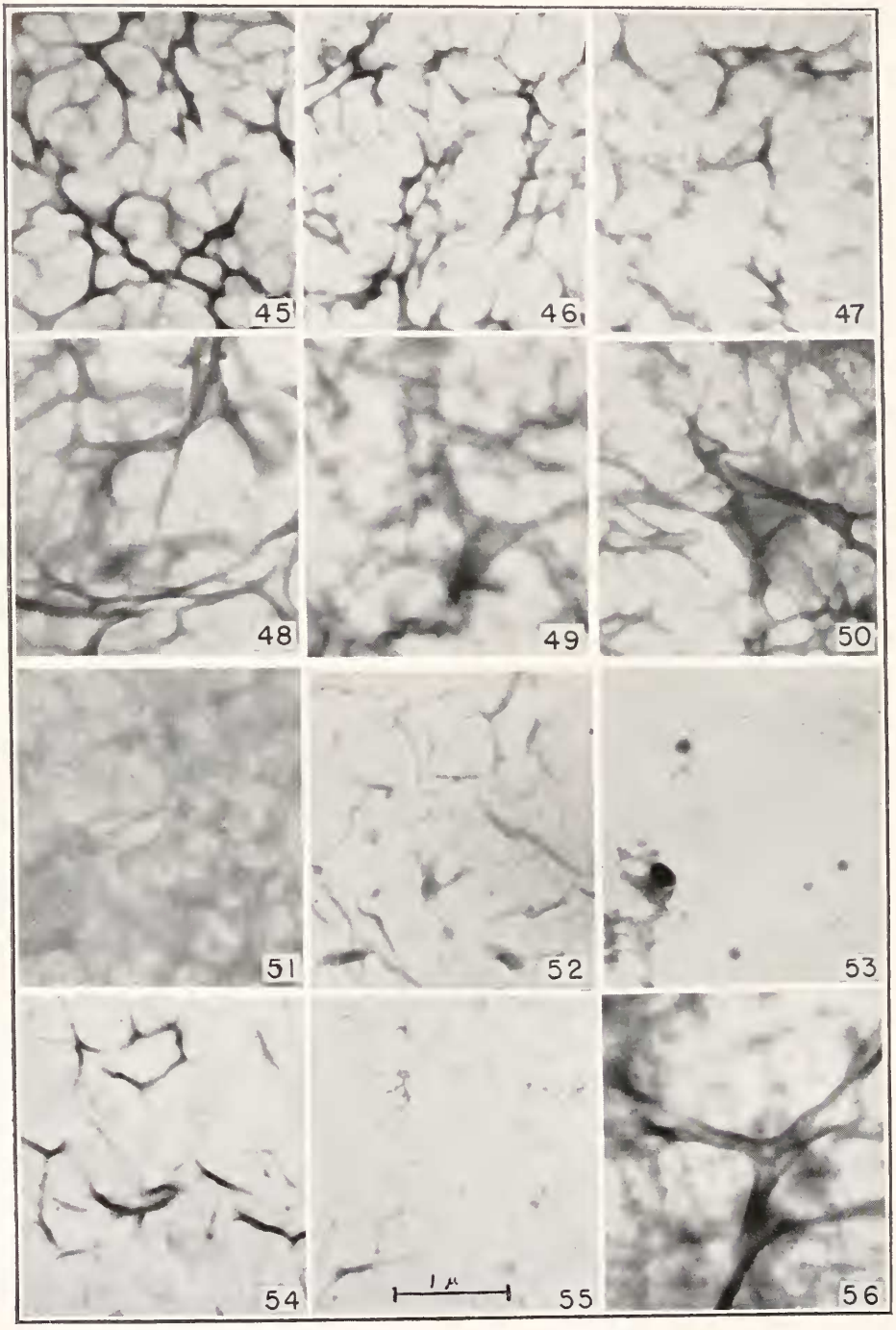
FIGURE 41. Mosquito larva, abdominal cuticle. Same treatment but washed only in 95 per cent ethyl alcohol. (No. 368d)

FIGURE 42. Mosquito larva, abdominal cuticle. Same treatment but washed only in water. (No. 367b)

FIGURE 43. Mosquito larva, abdominal cuticle. Same treatment but washed in water, then in 95 per cent ethyl alcohol. (No. 367d)

FIGURE 44. Mosquito larva, abdominal cuticle. Same treatment but washed in water, then in 95 per cent ethyl alcohol, then in water. (No. 368b)

PLATE V



loss of weight and no detectable change in electron micrographs. Soaking in water at high temperatures causes considerable loss in weight (at least largely removal of protein) and more or less extensive alteration of structure shown in electron micrographs.

All of the above examples are free from gross discontinuities (e.g., pore canals, gland ducts). The electron micrographs show that under favorable conditions changes that would invalidate permeability data do not occur at least down to the range of a few hundred Ångstrom units. However, most molecules of interest in studies of cuticle permeability are much smaller than this. Electron microscope studies show that numerous procedures introduce alterations that invalidate the use of such treatments of isolated cuticle but resolution in electron micrographs of cuticle is not fine enough to permit saying that no significant alterations occur after those treatments for which we could detect no changes. Other tests should also be used (e.g., change in membrane potential on soaking, etc.) and even with all possible checks final validation of the precise measurements possible with isolated cuticle will have to depend both on absence of demonstrable change and integration of the results with data from intact animals.

Discussion

The purification of chitin in membranes usually leads to extensive structural alterations demonstrable by electron microscopy. It follows that such purified chitin membranes can have no real biological significance in permeability studies. It also follows that we cannot continue to hold the old view that arthropod cuticle consists of a framework of chitin in which other components are merely embedded (see Fraenkel and Rudall, 1940, 1947; Richards, 1947 a, b).

Of more interest is the unexpected degree of dissimilarity of chitin patterns given by different types of cuticular membranes. The pattern obtained in a specific case is primarily correlated to the type of cuticle used rather than to the chemical treatment, and results of chitin purifications are reproducible with reasonable quali-

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Plate V

Honey bee (*Apis mellifica*), abdominal air sac. All figures reproduced at 16,000 ×

Figure 45. Normal, washed quickly in distilled water. (No. 229a)

Figure 46. Soaked in distilled water at 25° C. for 2 days. (No. 238e)

Figure 47. Soaked in isotonic saline at 25° C. for 2 days. (No. 253c)

Figure 48. Refluxed with boiling chloroform for 2 hours. (No. 228e)

Figure 49. Soaked in distilled water at 65° C. for 2 days. (No. 222a)

Figure 50. Soaked in distilled water at 65° C. for one week. Note differences between right and left halves indicative of more extraction from some areas than others. (No. 222e)

Figure 51. Soaked in distilled water at 100° C. for one day. (No. 323e)

Figure 52. Soaked in distilled water at 100° C. for 8 days. (No. 345b)

Figure 53. Soaked in distilled water at 100° C. for one month. Membrane so delicate that detectable in electron micrographs principally by small particle of debris on it. (No. 370d)

Figure 54. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 225c)

Figure 55. Refluxed with boiling chloroform for 2 hours, washed through acetone to water, and then treated with 10 per cent pepsin in acid solution at 36° C. for one day. (No. 228b)

Figure 56. Soaked in 0.02 per cent "Aerosol OT" (di octyl sodium sulfo succinate) in distilled water at 25° C. for 24 hours, washed in water. (No. 288a)

tative precision. Even though one can alter the pattern considerably after the purification treatment (alcohol versus water wash), we have not been able to obtain a single type of pattern from all types of cuticle by variations in technique. Depending on the type of membrane used, one obtains either a fibrous mat of greater or lesser degree of orientation, a vesicular membrane which is partly a fibrous feltwork, a reticulate membrane without distinct fibers or a seemingly homogeneous membrane with some small holes. If the continuous sheet in cockroach tracheae is found to be chitin we would have to add to this list a membrane homogeneous to less than 50 Å, at least when dry. These differences are not functions of membrane thickness and not functions of per cent de-acetylation of chitin to chitosan. Suggestions for an explanation of these diverse results would include: (a) a function of the relative percentage of chitin and protein in the membrane; (b) a function of degree of sclerotization; (c) a function of alkali-insoluble impurities; and (d) a function of different degrees of chitin polymerization. All four suggestions imply that chitin molecules vary either in size or in intermolecular bindings, and therefore they overlap and become aspects of one another.

The possibility of these diverse patterns being caused simply by variations in percentage of chitin present seems to us unlikely. There is really no convincing evidence for this statement. One can readily imagine variation in chitin percentage affecting the number of fibers per unit area, but we do not see how chitin percentage *per se* could account for fibrous networks versus homogeneous membranes, especially when the microfiber diameters (when found) are similar in different membranes, unless we also assume differences in cross linkages (which automatically moves the question to the fourth point).

Actually, the determination of the percentage of chitin present in any membrane is difficult, and we have only a crude idea of the accuracy of available methods (Campbell, 1929; Fraenkel and Rudall, 1947; Richards, 1947b). For most of the membranes studied in the present work it is not feasible to attempt such a determination. In current literature there are two opposed viewpoints on chitin-protein percentages in arthropod cuticle. Fraenkel and Rudall (1947) postulated a basically constant chitin percentage based on a hypothetical model of alternating monolayers of chitin and protein and assume that all deviations are secondary. Richards (1947) postulated a basically variable percentage of chitin based on the fact that all membranes have a high percentage of protein whereas the chitin percentage may range from 0 to 60 per cent. It seems to us difficult to reconcile our results with the alternating monolayer concept of Fraenkel and Rudall. The narrow range of microfiber diameters is readily interpretable on the idea of there being macromolecular chitin micelles which form mixed crystals with the protein molecules which have nearly identical lattice unit dimensions, but it would be surprising if monomolecular layers of chitin would reorient into macromolecular microfibers of such constant dimensions following removal of protein. Macrolayers in the cuticle are evident (e.g., interference color phenomena, alternating laminae in electron micrographs) but monolayers or even single micellar layers are not. At present all ideas are only working hypotheses.

The second possibility, variation in degree of sclerotization, is difficult to analyze. By definition, sclerotization is the formation of sclerites, i.e., hardened areas in the cuticle. Campbell (1929) was the first to show that this hardening is not due to chitin *per se*. Pryor (1940) elucidated part of the picture by demonstrating the

tanning of cuticular proteins by oxidized orthodihydroxyphenols with concurrent hardening. If this were the whole story of sclerotization the process should not affect the structure of purified chitin. But x-ray and other data lead to the assumption of chitin-protein bindings, the nature of which is not known. Our work shows that purification of chitin has less destructive effect on sclerotized membranes than on non-sclerotized membranes despite the fact that the sclerotized membranes contain lower percentages of chitin (Fraenkel and Rudall, 1947). It seems that as a result of sclerrotization the chitin molecule linkages are so changed that a different pattern is obtained following so-called purification of the chitin. Again we are led to the idea of variations in chitin cross-linkages to account for our diverse pictures even if the diversity is found to be a by-product of sclerotization.

The third suggestion, variable alkali-insoluble impurities, is at present only a conjecture. One of the great difficulties of work on cuticle is that we have no means of determining the degree of purification of chitin. It is quite possible that no one has ever seen pure chitin (note the low nitrogen value consistently obtained by Fraenkel and Rudall, 1947). Solution in acid leads to rapid hydrolysis of ether linkages (Clark and Smith, 1936) and it is not certain that solutions in lithium thiocyanate are free from impurities. Until we have conclusive analyses there is little that can be done about this uncertainty.

The fourth suggestion, various degrees of polymerization of chitin molecules, seems to fit our diverse pictures best but it cannot be claimed that our data prove this to be true. Chitin, unlike cellulose, is not known to occur naturally in anything approaching the pure state. It is customarily assumed that there must be chitin cross-linkages to or through other compounds but the possibility of various degrees of cross-linkages between chitin molecules seems not to have been suggested—no doubt largely because nothing definite is known about it. When microfibers are obtained they show a rather narrow range of diameters irrespective of whether they parallel one another or run randomly. This suggests there is some real significance to the values (micelle diameters ?), but the absence of such microfibers in certain purified membranes would seem to necessitate a different type or degree of cross-linkages between chitin chains or chitin micelles.

To account for our diverse electron microscope pictures there must be different degrees or kinds of reorientation of chitin molecules following removal of other membrane components. Unfortunately we can do no more than speculate about this at present. Conceivably such results might be a function of chitin chain lengths (if these vary like cellulose chains), or of stable cross-linkages present before purification, or of different spatial patterns in the cuticle tending to different degrees of chain cross-linkages (hydrogen bonds ?) following purification, or of variable amounts of stabilizing impurities if such exist. Apparently these linkages would not involve the acetyl group since purified chitin and chitosan membranes give similar electron micrographs. Obviously we need to know more about chitin chemistry on the polymer level.

Summary

1. Chitin purifications alter the structure of cuticular membranes of insects more or less extensively.

2. Our data agree with the x-ray diffraction studies of Fraenkel and Rudall in showing:

(a) The cuticle cannot be viewed as a rigid chitin framework in the interstices of which other components are deposited.

(b) Protein extraction methods have no value for localization of membrane components.

(c) Purified chitinous membranes have no significance for studies on the permeability of arthropod cuticle.

3. Several but not all types of membranes after purification yielded microfibers of chitin which after drying have diameters of $<$ 100 to 300 Å ($<$ 0.01 to 0.03 μ). It is suggested that these values may represent chitin micelle diameters.

4. Unexpected diversity in chitin patterns was obtained. Possible interpretations are discussed, and it is suggested that chitin cross-linkages vary considerably from one type of membrane to another type.

5. Chloroform and acetone remove the lipid epicuticle without affecting the structure of the underlying protein epicuticle and endocuticle. Detergents disrupt both the lipid epicuticle and underlying protein layers.

6. The effects of water and salt solutions on the structure of isolated cuticular membranes depend on temperature, the salt used and its concentration and the type of membrane. There is good correlation between weight losses recorded at various temperatures by Fraenkel and Rudall and structural changes found in our work. It is concluded that isolated cuticular membranes that are free from gross discontinuities can be used to a certain extent in permeability studies employing methods of physical chemistry but only when parallel tests show that the particular membranes remain reasonably near their original structure and composition.

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